Crosstalk between B lymphocytes, microbiota and the intestinal epithelium governs immunity versus metabolism in the gut

N. Shulzhenko and A. Morgun et al.

Supplementary Data

The *Lactococcus* genus subgroup F49WGKH02HS7CN shown in Fig. 3b is represented by the following sequence:

rRNA <1..>232

/product="16S ribosomal RNA"

>F49WGKH02HS7CN<

>TTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTCGGCTATGTATC
ATCGCCTTGGTAGTCCATTACACTACCAACTAGCTAATACAACGCGGGTCCATCTCT
TAGTGCACCAATTGGTGCTTTCAAATCACTAACATGTGTTAGTGATTGTTATGCGGT
ATTAGCTATCGTTTCCAATGTTCGTCCCCCGNTAAGAGGTAGGTTACCCACGCGTTA
CTCC<

Supplementary Methods

<u>Organ dissection and collection of samples</u>

Small intestines were harvested in ice-cold PBS, folded in half lengthwise on a paper towel. About 2 cm of the middle part were dissected from surrounding fat (avoiding Peyer's patches), flushed with cold PBS and immediately frozen on dry ice for RNA isolation. The content of the adjacent 5 cm was squeezed into a sterile tube for DNA isolation. B lymphocytes were isolated from spleens using Mouse CD45R (B220) MicroBeads (Miltenyi Biotech). For isolation of intestinal epithelium, ~10 cm of the harvested jejunum were open longitudinally, rinsed twice with ice-cold HBSS and cut into 5 mm pieces into HBSS with 0.2% BSA and 5mM EDTA. The tissue was incubated at 37oC for 30 min with continuous stirring, after which the cell suspension was filtered and washed. Perigonadal fat was dissected around testis or uterus.

<u>Microarray sample preparation and hybridization</u>

Total RNA was isolated using TRIzol (Invitrogen) or RNAeasy (Qiagen) following manufacturers' protocols. For mouse tissues, 20µg of total RNA were fluorescently labeled in a reverse transcription reaction using 2µl Cy3-dUTP or Cy5-dUTP (GE Healthcare), 1 µg Oligo(dT)12-18 Primer, 0.5mM of each dATP, dCTP, dGTP, 0.15mM dTTP, 10mM DTT, 1.5µl SuperScript II

reverse transcriptase and 0.5µl RNAse inhibitor (all from Invitrogen). Obtained cDNA was purified using Vivaspin 500 columns (10,000 MWCO, Sartorius) and TE buffer. Using dye-swap direct microarray design, corresponding experimental (knockout) and control samples were co-hybridized using MAUI hybridization system to the arrays printed by the NIAID Microarray Facility or by Agilent. For human RNA, 50ng was amplified and labeled using Aminoallyl RNA Amplification and Labeling System (NuGEN Technologies) following manufacturer's protocol. Cy5-labeled samples were co-hybridized with a common reference RNA from normal duodenum (Clontech) labeled with Cy3 to Agilent Whole Human Genome Microarrays. Images were scanned by GenePix4000B Scanner (Axon Instruments/Molecular Devices) or Agilent Scanner.

Microbiome

Bacterial universal primers 27F and 338R were used for PCR amplification of the V1-V2 hypervariable regions of 16S rRNA genes using 50 ng of the template DNA (isolated from jejunal contents) in a total reaction volume of 50uL. The 338R primer included a unique 8nt sequence tag to barcode each sample. Equimolar amounts (100ng) of the PCR amplicons were mixed in a single tube. The amplicon mixtures were sequenced on 454 FLX pyrosequencing platform. Raw 16S sequences and flowgram (SFF files) were deposited in NCBI Short Read Archive (SRA) under the accession number SRA023521. Sequences obtained from 454 pyrosequencing were binned by using the sample-specific barcode sequences and trimmed by removal of the barcode and primer sequences.

Leptin measurement
Serum leptin was measured using Mouse leptin Quantikine kit (R&D Systems) or LINCOplex Biomarker Panels (Millipore).

LPS measurement
Serum LPS was measured using Limulus Amebocyte Lysis chromogenic endpoint assay as per manufacturer's instructions (Hycult Biotech).

Cholesterol and dietary fat absorption assays
Cholesterol and dietary fat absorption in mice was performed by the Cincinnati Mouse Metabolic Phenotyping Center as described in Battle et al, 2008. Briefly, radioactive ^{14}C-cholesterol and ^{3}H-sitostanol were gavaged into adult male mice that were fasted overnight. Extracts from fecal samples taken 24 hours post-gavage were measured for ^{14}C and ^{3}H radioactivity by scintillation counting, and dietary and fecal ratios of the isotopes were calculated. Dietary fat absorption was determined using a diet modified to 16% fat [butter] of which 5% was sucrose polybehenate. Fecal pellets from day 4 on the experimental diet were analyzed.

Body composition was measured in non-anesthetized mice using the Echo3-in-1 NMR analyzer (Echo Medical Systems, Houston, TX).

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Supplementary Figures

a

B lymphocyte knockout and control mice

Mice with deletion of the transmembrane segment of IgM: no mature B cells, no IgM, IgG, IgA

Kitamura et al., *Nature* 1991
Shulzhenko et al., *Genes and Immunity* 2009

Littermates

B10.AμMT-/-   **B10.AμMT+/-**

12 pairs

Non-littermates

BALB/c JhKO   **BALB/c WT**   **B10.AμMT-/-**   **B10.A WT**

Mice with deletion of J segment of IgM gene: no mature B cells, no IgM, IgG, IgA

Chen J et al., *International Immunology* 1993

10 pairs   *5 pairs*

From all mice: Take jejunum → Isolate RNA → Microarrays

b
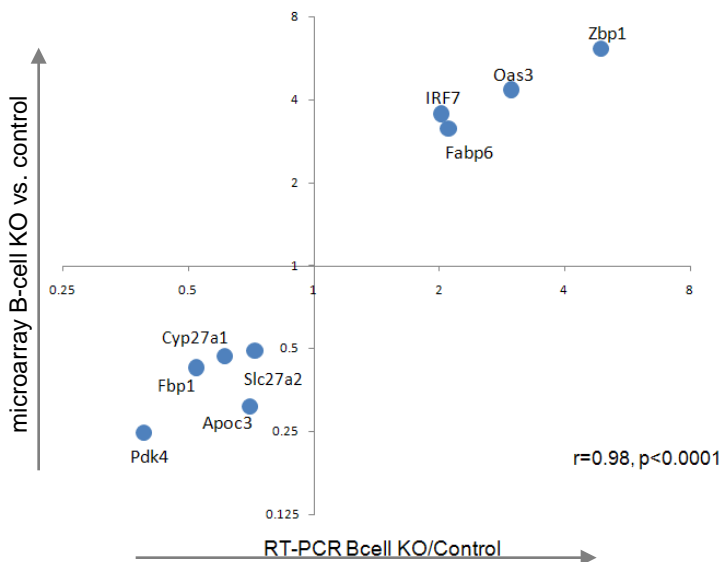
Supplementary Figure 1. Defining and validating the B cell KO profile. **(a)** Design of the experiment that was used for defining the gene expression B-cell KO profile in the small intestine. **(b)** Analysis of consistency between measurements of gene expression performed by microarrays and RT-PCR for the indicated genes from BcKO profile.

a

Interferon-inducible genes in the Up-regulated genes of B-cell KO profile (~30% of profile)

b

c

Serum LPS
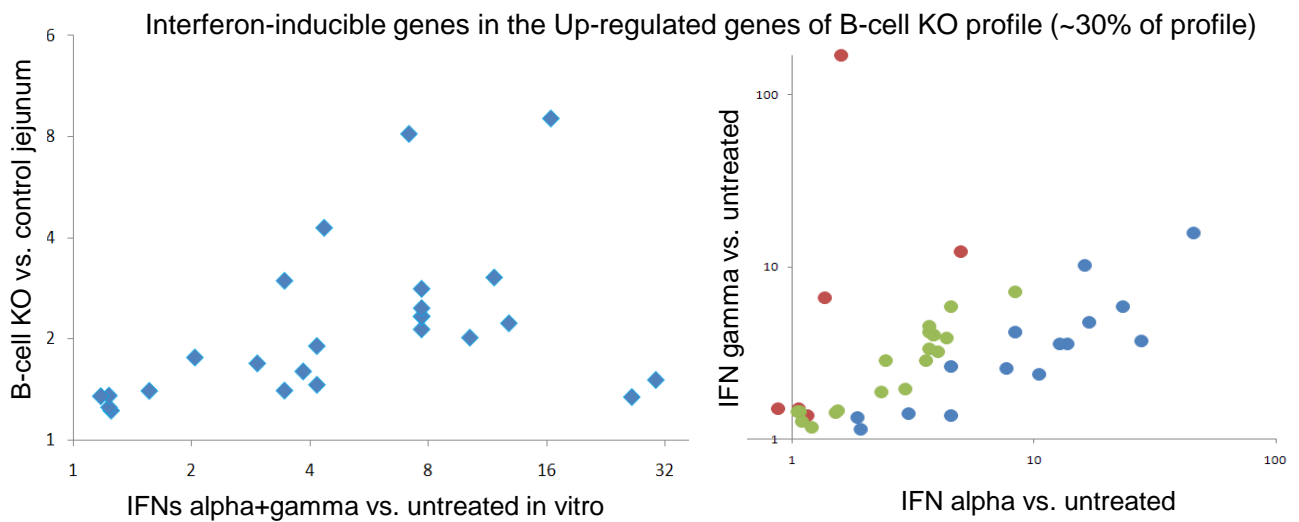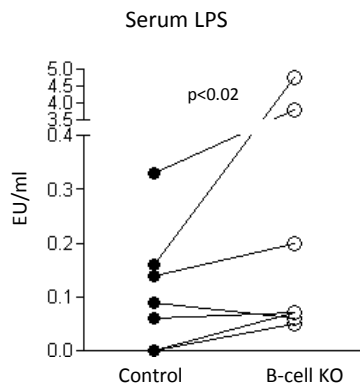
Supplementary Figure 2. Relation of BcKO expression profile to interferon, gut morphology and LPS levels in BcKO mice.

(a) IFN-inducible genes among the up-regulated genes in B-cell KO profile. Gene expression in the jejunum of B-cell KO mice correlated (r=0.55, p<0.01) to the expression in IFN- alpha+gamma treated cell line (left panel) . Predominance of IFN-alpha for the induction of up-regulated genes in B-cell KO profile (right panel: red genes , IFN-gamma-dependent; blue, IFN-alpha; green, either IFN-dependant.) Microarray data for IFN treated cells was taken from GDS2341.

(b) Histology (H&E) of jejunum in B-cell KO and control heterozygous mice. No difference was noticed upon blind evaluation. Representative images of 4 mice per group.

**(c)** Serum levels of LPS in 7 pairs of B-cell KO and littermate control mice . Nature Medicine doi:10.1038/nm.2505
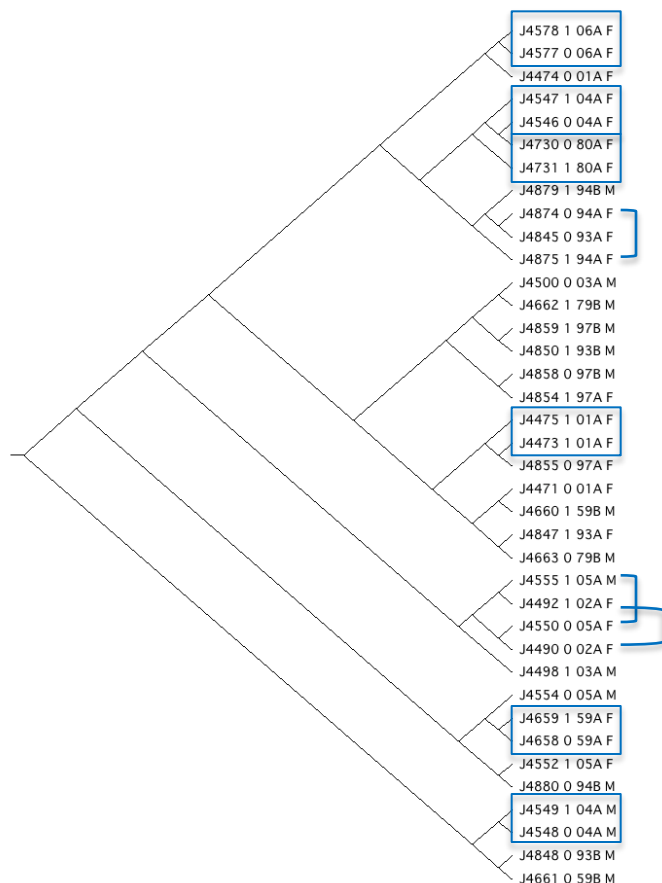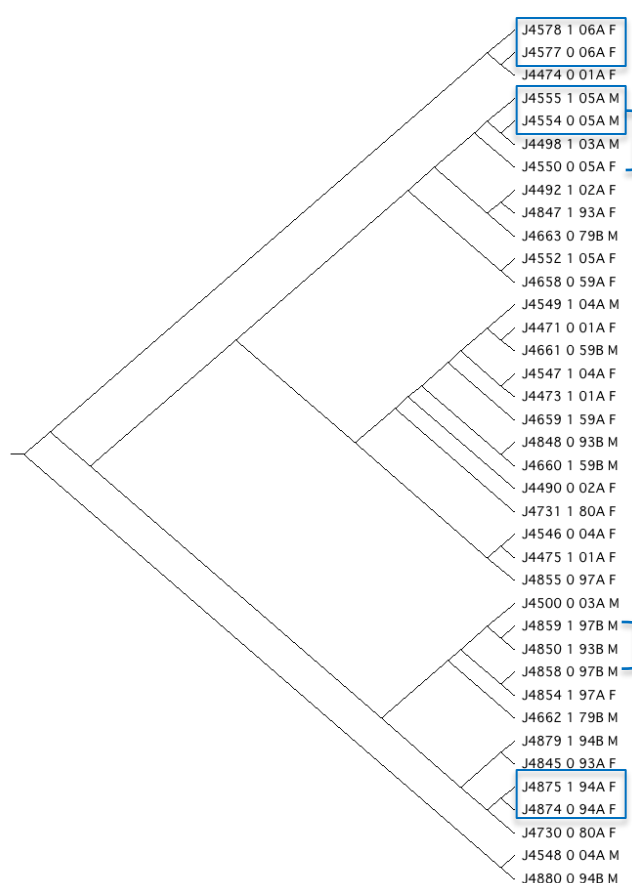
a

Supplementary Figure 3. Analysis of microbiota of jejunum content from BcKO and control mice. **(a)** Similarities of microbiota among the samples based on either the Jaccard similarity measure (Jaccard) or the YCtheta similarity measure (Yctheta) using the Mothur approach. Operational taxonomic unit (OTU) cutoff of 0.05 (95% sequence identity) was used to generate these dendrograms. The Jaccard similarity coefficient considers the presence and absence of shared OTUs in each sample to create a similarity matrix based on shared membership. Jaccard measure does not take abundance information into account. The YCtheta similarity measure incorporates the OTU abundance information and therefore provides a measure of the community structure. The litters appear to cluster together more frequently in Jaccard tree than in YCtheta tree. This observation suggests that the membership of the microbiota inherited from the parent is more stable than the relative abundance of each OTUs (species), which can naturally fluctuate over time. Clustering by litter (denoted with boxes and brackets above) is the only consistent trend we observed. We did not observe consistent clustering of samples by sex or by host genotype (KO vs. HET) using the Mothur approach (depicted) or other approaches such as clustering by known taxa (RDP Classifier results) or principle component analysis of RDP Classifier results (in house SAS scripts), or principle coordinate analysis using Unifrac (data not shown). The first symbol (e.g. J4661) represents mouse ID , the second one represents genotype (0-KO, 1-control), the third sysmbol is litter ID (e.g. 04A), the last is gender (M-male, F-female).

b
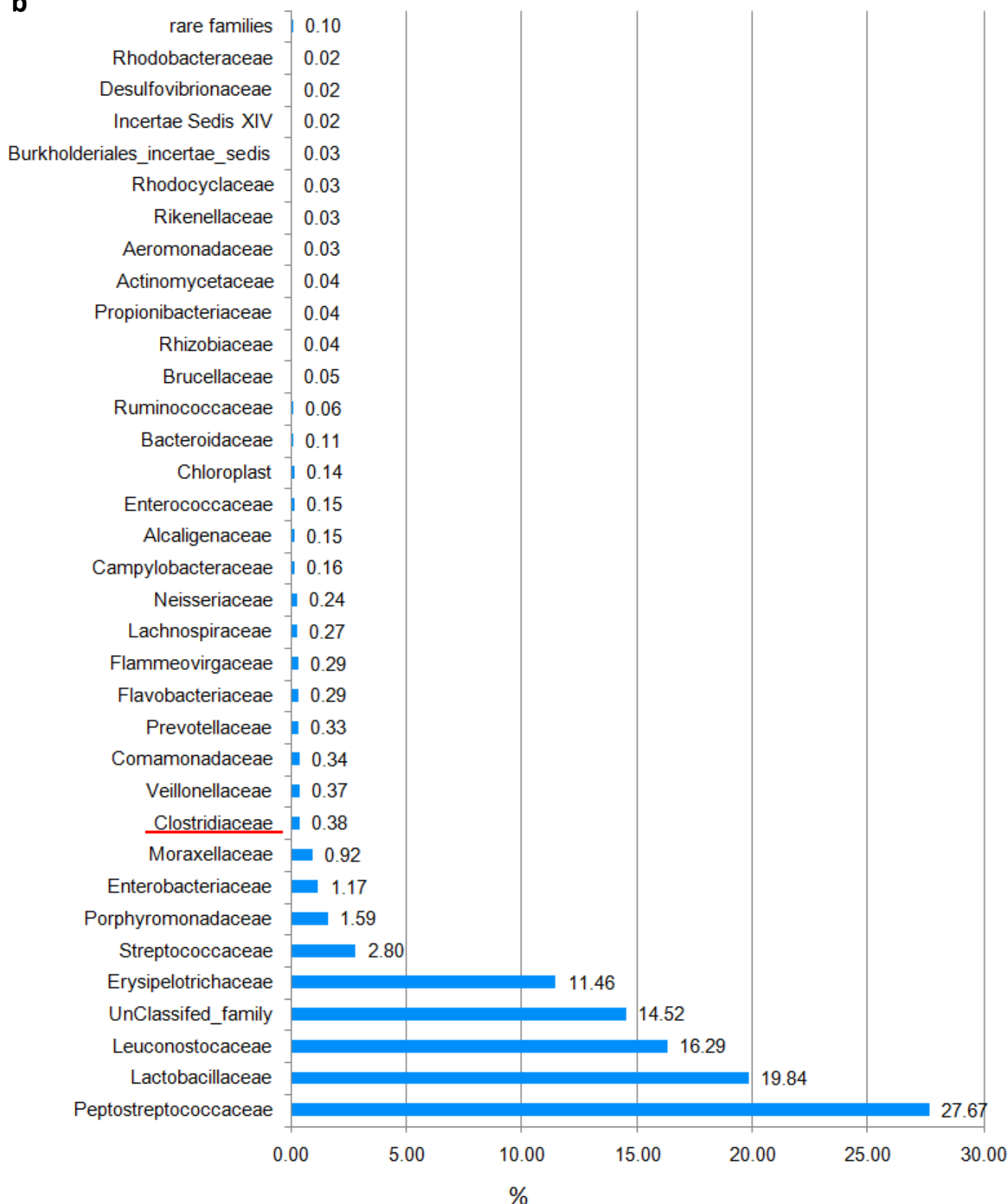
**Supplementary Figure 3.** Analysis of microbiota of jejunum content from BcKO and control mice.

(b) Relative abundance (%) of different microbiota families in jejunum content. The values were calculated by dividing the total number of reads assigned to a given family by total number of reads in a sample. Data represents average of 19 control mice.

a

b
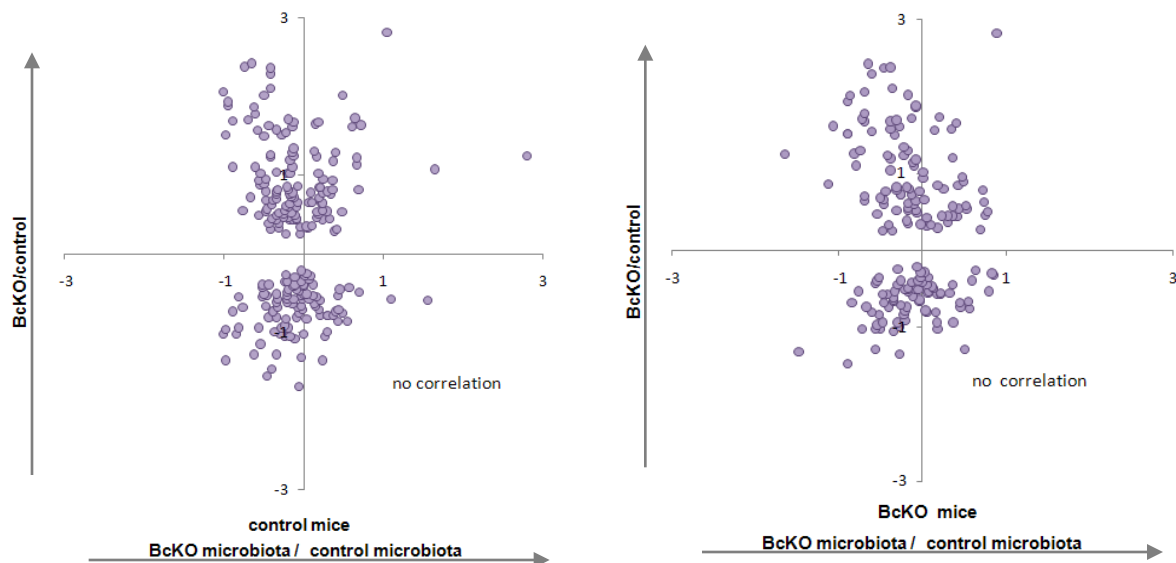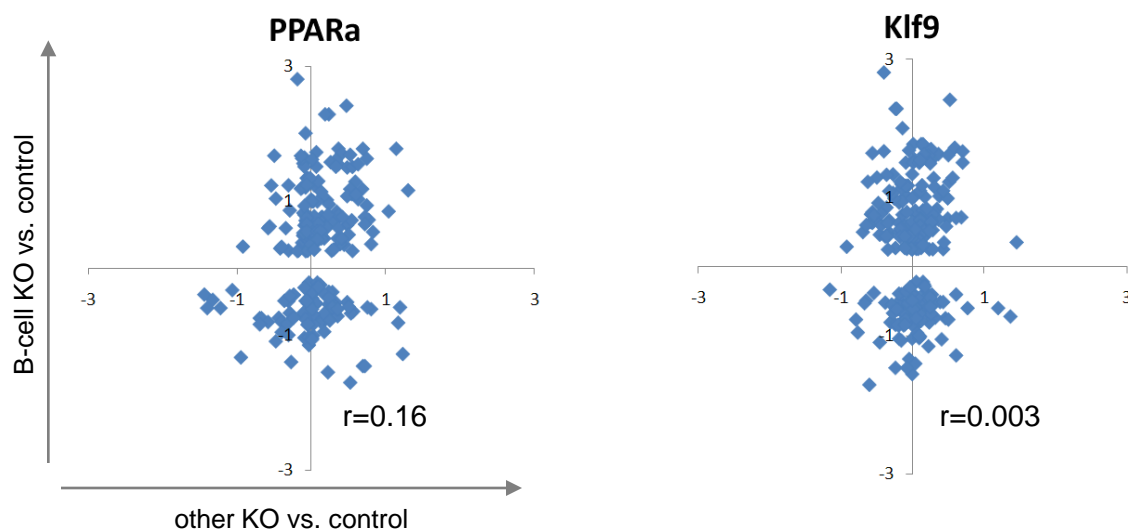
Supplementary Fig. 4. BcKO profile in mice colonized with different microbiota and in mice with intestinal gene knockouts**.**

(a) No difference in expression for the genes of BcKO profile between germ-free mice of the same genotype ~3 weeks after colonization with microbiota from control or B cell KO mice.

(b) Comparison of gene expression in jejunum between B-cell KO and Ppara and Klf9 KO. Each symbol is a gene from B-cell KO profile.
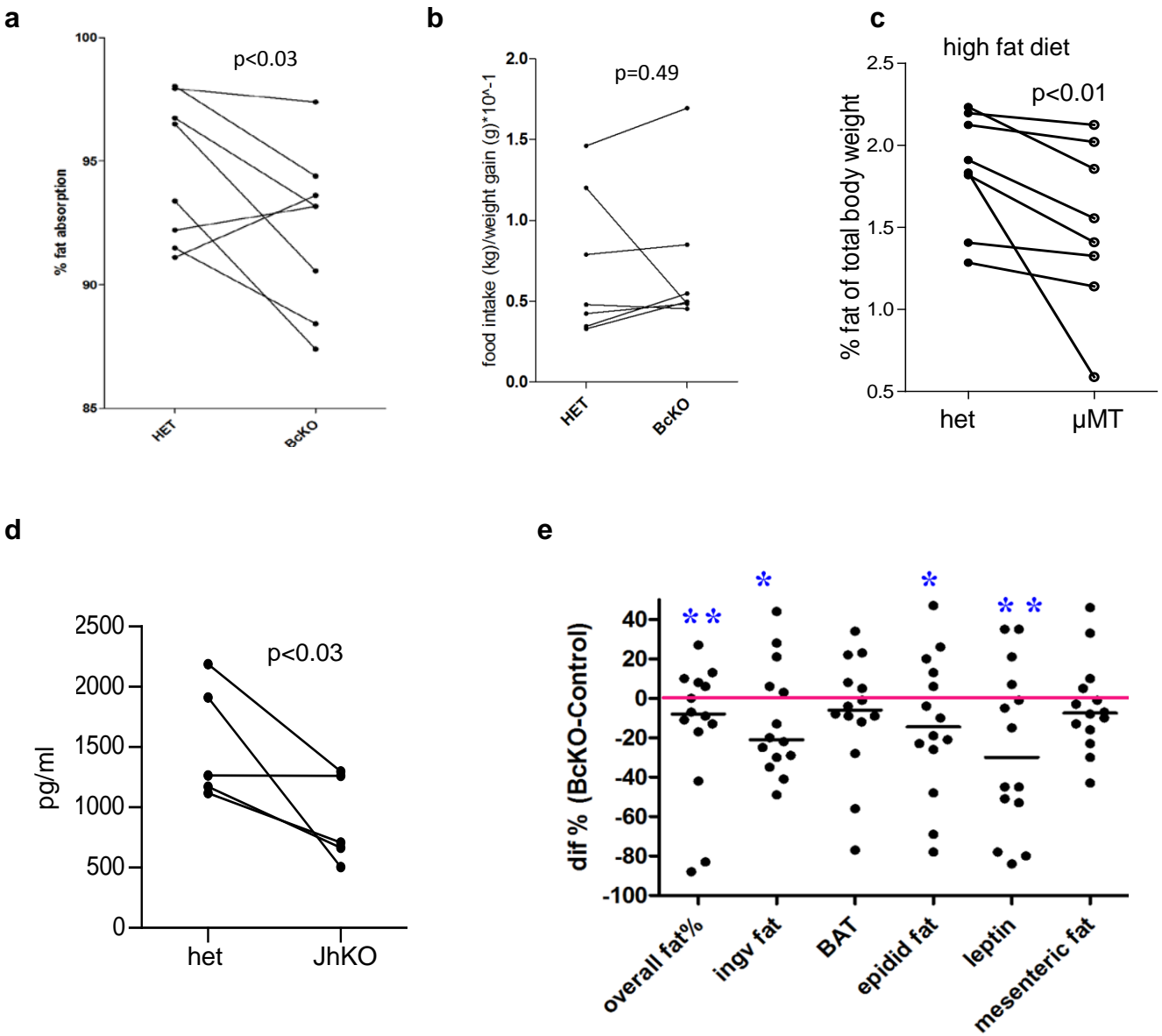
Supplementary Figure 5. Metabolic markers in BcKO mice.

(a) Comparison of fat absorption and **(b)** food intake between BcKO and their littermate controls.

(c) Proportion of perigonadal fat (g) in relation to total body weight (g) in B-cell KO, their corresponding control mice on <u>high fat diet</u>. Each dot represents median value of each genotype in a given litter. Each line represents one litter.

(d) Serum leptin levels in JhKO and their corresponding control mice. Each dot represents the median value of each genotype in a given litter. Each line represents one litter.

(e) Comparison of different fat stores between B cell KO and their littermate controls in an additional group of mice (number of litters 14, number of mice ~50, ** $p<0.06$; * $p<0.1$). Each dot represent one litter. *Ingv fat*, inguinal fat; *BAT*, brown fat; *epidid*, epididimal

a
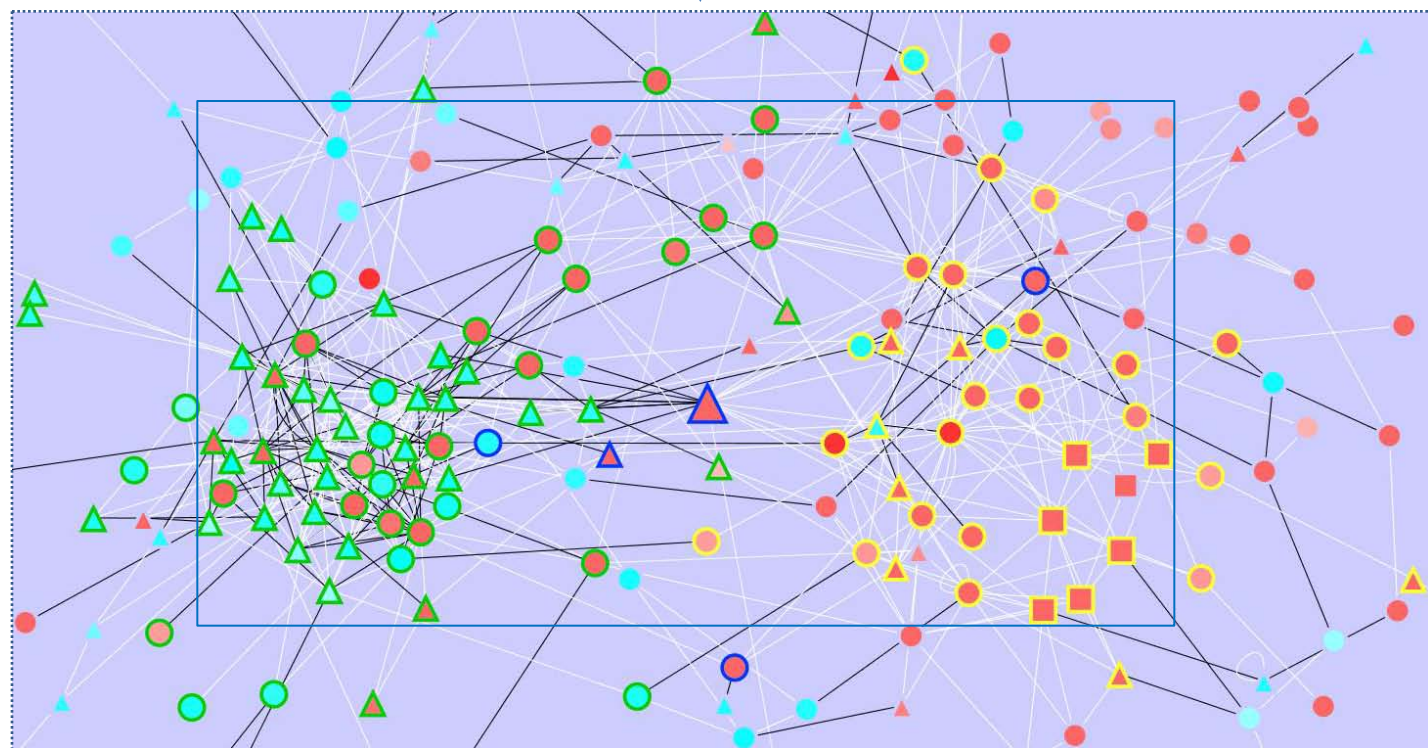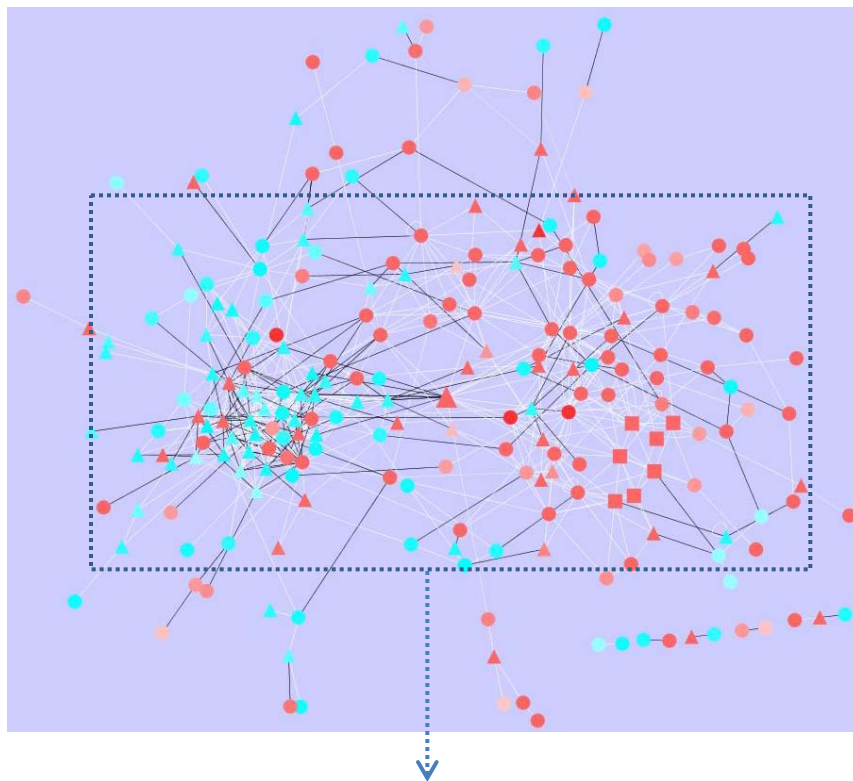
Supplementary Figure 6. Gene expression network of genes from the B-cell KO profile. **(a)** The network was reconstructed based on the data of 39 control mice. Upper panel: the whole network (228 genes, 688 connections). The lower panel is the zoom in the area delineated by the rectangle in the upper panel. Each line represents a correlation; each node represents a gene; line-colors: white are positive and black are negative correlations; node-shapes: triangles are Gata4-dependent genes; squares are T cell genes; circles are all other genes; node-fill-colors: Red is up-regulation; Light blue is down-regulation; node-outline-colors: green is a sub-network #1; yellow is a subnetwork #2.

The rectangle in the lower panel outlines the area shown in the main Fig. 5.

b

	GO Identifier	GO Term	Ontology ▲	p-value
☐	GO:0055114	oxidation reduction	Biological process	3.52806e-06
☐	GO:0034097	response to cytokine stimulus	Biological process	0.000235958
☐	GO:0006869	lipid transport	Biological process	0.000530657
☐	GO:0009725	response to hormone stimulus	Biological process	0.000592045
☐	GO:0009719	response to endogenous stimulus	Biological process	0.00107046
☐	GO:0045087	innate immune response	Biological process	0.00117055
☐	GO:0009615	response to virus	Biological process	0.00131375
☐	GO:0034754	cellular hormone metabolic process	Biological process	0.00131375
☐	GO:0043434	response to peptide hormone stimulus	Biological process	0.00251943
☐	GO:0008202	steroid metabolic process	Biological process	0.00280296
☐	GO:0032787	monocarboxylic acid metabolic process	Biological process	0.00297836
☐	GO:0042445	hormone metabolic process	Biological process	0.00398287
☐	GO:0046942	carboxylic acid transport	Biological process	0.00438023
☐	GO:0015849	organic acid transport	Biological process	0.00451783
☐	GO:0006952	defense response	Biological process	0.0061211
☐	GO:0006955	immune response	Biological process	0.00789177

c

	GO Identifier	GO Term	p-value ▲
☐	GO:0006955	immune response	6.5096e-10
☐	GO:0002376	immune system process	1.34954e-07
☐	GO:0043235	receptor complex	9.34475e-06
☐	GO:0034097	response to cytokine stimulus	3.21112e-05
☐	GO:0006954	inflammatory response	7.05896e-05
☐	GO:0050896	response to stimulus	0.000105932
☐	GO:0005126	cytokine receptor binding	0.000113569
☐	GO:0009611	response to wounding	0.000426864
☐	GO:0002253	activation of immune response	0.000685069
☐	GO:0010033	response to organic substance	0.00104234
☐	GO:0006952	defense response	0.00147558
☐	GO:0002250	adaptive immune response	0.00154974
☐	GO:0002460	adaptive immune response based on somatic recombin	0.00154974
☐	GO:0002449	lymphocyte mediated immunity	0.00166149
☐	GO:0004866	endopeptidase inhibitor activity	0.00173872
☐	GO:0050778	positive regulation of immune response	0.00202664
☐	GO:0030414	peptidase inhibitor activity	0.00207005
☐	GO:0002443	leukocyte mediated immunity	0.00220373
☐	GO:0005576	extracellular region	0.00221989
☐	GO:0004252	serine-type endopeptidase activity	0.00253623
☐	GO:0048584	positive regulation of response to stimulus	0.00365182
☐	GO:0004175	endopeptidase activity	0.00371914
☐	GO:0009605	response to external stimulus	0.00406433
☐	GO:0002252	immune effector process	0.004375

Supplementary Figure 6. Gene expression network of genes from the B-cell KO profile.
(b) Enrichment of Gene Ontology categories for the genes from the subnetwork # 1 (b) and
subnetwork #2 **(c)**
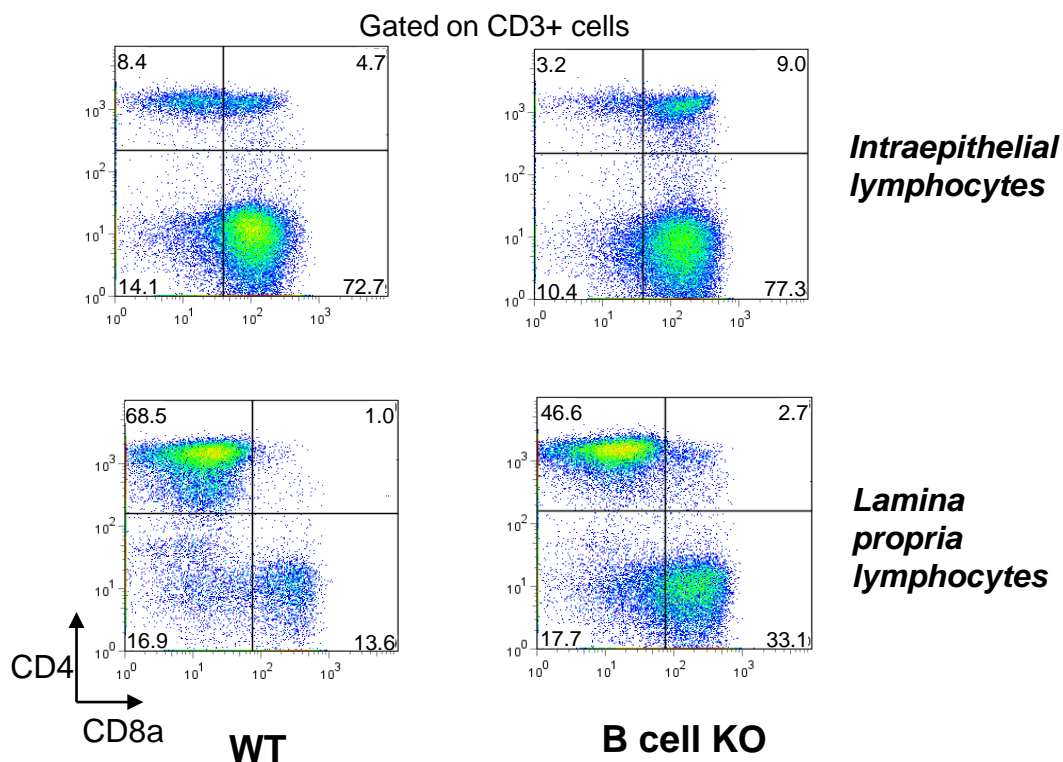
Supplementary Figure 7. Increased proportion of CD8+ T lymphocytes in the gut of B-cell KO mice. Note the higher proportion of CD4/CD8 double positive T cells in the intraepithelial lymphocytes and CD8 positive cells in the lamina propria of B-cell KO mouse.
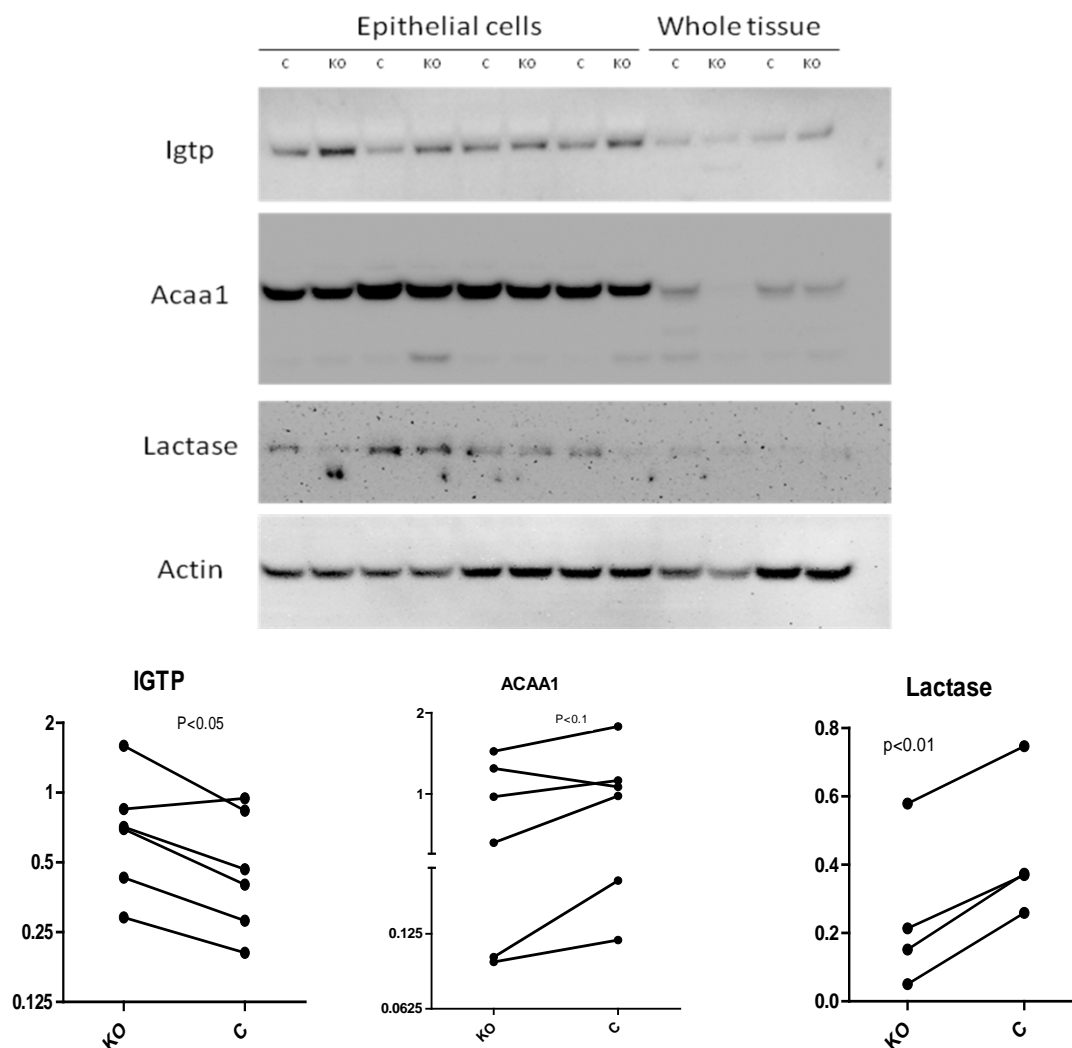
Supplementary Figure 8. Protein levels in epithelial cell and whole tissue lysates from jejunum of BcKO and control mice. The levels of proteins were assayed by Western blot and quantified by densitometry and normalized to the levels of actin (graphs). Levels of Lactase were quantified only in epithelial cells lysates. Antibodies used: IGTP (Santa Cruz # sc-11084), ACAA1 (Sigma, HPA007244); Lactase (Acris , AP10118PU-N).

genes/stimuli	CpG	Ecoli	IFN	LPS	Poly I:C
IRF7	ns	<0.001	<0.0001	<0.001	0.017
Gbp6	ns	<0.01	<0.0001	<0.001	ns
Zbp1	ns	<0.001	<0.0001	<0.001	0.0042
Acaa1b	ns	0.02	0.0073	0.2	0.0033
Crip1	0.0033	0.0017	0.0032	0.042	ns
Cyp27a1	ns	0.002	0.0032	0.047	ns
Dbp	0.042	<0.001	0.0053	<0.001	0.0027
Pdk4	ns	0.2	0.017	0.24	0.0417
Vat1	ns	0.47	0.0032	0.24	0.2

Supplementary Figure 9. P values for comparison between treated and untreated MODE-K cells for the experiment shown in Fig. 6A. p values < 0.05 have background (Orange corresponds to up-, blue to down-regulated genes, gray for regulation opposite to the original BcKO profile.
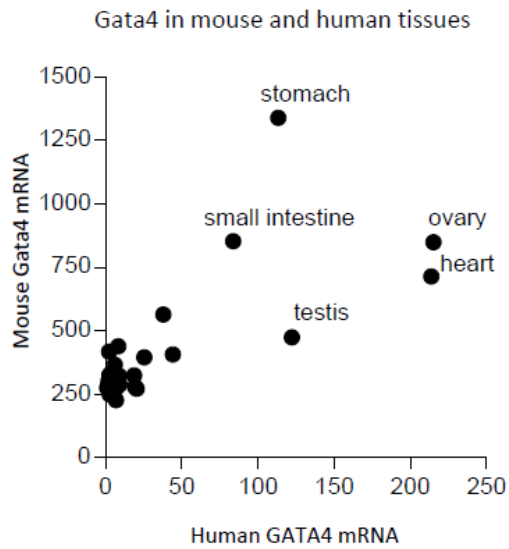
Supplementary Figure 10. Expression of GATA4 gene in different mouse and human tissues. Data was extracted from publicly available databases BioGPS (www.biogps.gnf.org) and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GDS1096 .